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Marine transmissible cancer navigates urbanised waters, threatening to spillover

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1 **Marine transmissible cancer navigates urbanised waters, threatening to spillover**

2

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12

13 **Abstract**

14 Transmissible cancer, a unique form of microparasites that spreads through direct
15 transmission of living cancer cells, is increasingly reported in marine bivalves. In this study,
16 we sought to understand the ecology of the propagation of *Mytilus trossulus* Bivalve
17 Transmissible Neoplasia 2 (MtrBTN2), a transmissible cancer affecting four *Mytilus* mussel
18 species worldwide. We investigated the prevalence of MtrBTN2 in the mosaic hybrid zone of
19 *M. edulis* and *M. galloprovincialis* along the French Atlantic coast, sampling contrasting
20 natural and anthropogenic habitats. We observed a similar prevalence in both species, likely
21 due to the proximity of the two species in this region. Our results showed that ports had
22 higher prevalence of MtrBTN2, with a hotspot observed at a shuttle landing dock. No cancer
23 was found in natural beds except for two sites around the hotspot, suggesting spillover. Ports
24 may provide favourable conditions for the transmission of MtrBTN2, such as high mussel
25 density, confined sheltered shores, or buffered temperatures. Ships may also spread the
26 disease through biofouling, with maritime traffic being the best predictor of MtrBTN2
27 prevalence. Our results suggest ports may serve as epidemiological hubs, with maritime
28 routes providing artificial gateways for MtrBTN2 propagation. This highlights the

29 importance of preventing biofouling on docks and ships' hulls to limit the spread of marine
30 pathogens.

31

32 **Keywords:** Transmissible cancer, Bivalve Transmissible Neoplasia, epidemiology,
33 biofouling, spillover, *Mytilus* mussels

34

35 **1. Introduction**

36

37 Transmissible cancers are malignant cell lineages that have acquired the ability to
38 infect new hosts through the transmission of living cancer cells. Eleven transmissible cancer
39 lineages have been described to date: one in dogs (canine transmissible venereal tumour,
40 CTVT [1, 2]), two in Tasmanian devils (devil facial tumour, DFT1 and DFT2 [3, 4]), and
41 eight in different marine bivalve species (bivalve transmissible neoplasia, BTNs [5–9]).
42 While direct contact is necessary for CTVT and DFTs transmission, via coitus for the former
43 and biting for the latter, the transmission of BTNs between sessile shellfish is assumed to
44 occur through the water column [10, 11]. *Mytilus* spp. mussels are affected by two BTN
45 lineages known as MtrBTN1 and MtrBTN2 that originate from two different *M. trossulus*
46 founder hosts. MtrBTN2 is distributed worldwide and has crossed the species barrier several
47 times to infect four *Mytilus* species: *M. trossulus* in East Asia [12, 13], *M. chilensis* in South
48 America [9], and *M. edulis* and *M. galloprovincialis* in Europe [9, 14]. The oldest MtrBTN2
49 sample is 14 years old. Genetic data suggest that it could be at least one or two orders of
50 magnitude more ancient, although dating is difficult due to an apparent acceleration of the
51 mitochondrial clock [12, 14].

52

53 The global distribution of the MtrBTN2 lineage remains enigmatic [9]. The other known
54 transmissible cancer with such a worldwide distribution is CTVT in dogs. CTVT emerged
55 4,000 to 8,000 years ago, probably in Eastern Asia, and has expanded rapidly worldwide over
56 the past 500 years, probably facilitated by the development of maritime transportation [15].
57 Transmission of MtrBTN2 within mussel populations occurs presumably through filter-
58 feeding (discussed in [10, 16]). Cancer cells can survive for several days in seawater [10],
59 which greatly increases their chances of infecting new hosts. However, it is unlikely that
60 transport of cancer cells by marine currents alone explains such a global distribution. Indeed,
61 just as gamete dispersal effectiveness is limited to a few metres in free-spawning
62 invertebrates [17], the dilution of cancer cells in the marine environment may strongly limit

63 the success of transmission over long distances (even locally in a mussel bed). Shipping
64 traffic has been proposed as the most likely explanation for the global distribution of
65 MtrBTN2 -i.e., the transport of disease-carrying mussels on ship hulls [9, 12]. To our
66 knowledge, these claims have remained speculative and no formal evidence of the effect of
67 shipping traffic and port habitat on the epidemiology of BTN has been provided to date.

68

69 European mussels are affected by MtrBTN2, with a much higher prevalence in *M. edulis* (of
70 the order of 1/100) than in *M. galloprovincialis* (of the order of 1/1000) [14]. These two host
71 species are parapatric in Europe and can coexist in contact zones where hybridisation is
72 taking place [18–20]. Associations between genetic backgrounds and environmental variables
73 indicate that the environment partly influences the structure and maintenance of the hybrid
74 zones: in the study area, *M. edulis* genotypes are more frequent in sheltered habitats under
75 freshwater influence, while *M. galloprovincialis* genotypes are more frequent in habitats
76 exposed to wave action and ocean salinities [21, 22]. In this context, the distribution and
77 propagation of MtrBTN2 is likely to be influenced by host genetic background and
78 population composition, as well as by other environmental factors such as pollution,
79 population density and abiotic environmental variables (e.g., salinity, temperature, pH).
80 However, little is known about the ecology of MtrBTN2 in relation to its host and the
81 possible environmental factors influencing the epidemiology of this transmissible cancer.

82

83 In the present study, we investigated the occurrence of MtrBTN2 in a survey area in Southern
84 Brittany (France) which has contrasting natural and anthropogenic habitats and a mosaic
85 distribution of *M. edulis* and *M. galloprovincialis*. We sampled 40 natural populations as well
86 as 9 mussel farms, 7 floating buoys, and 20 ports. To detect MtrBTN2-infected mussels, we
87 developed and performed a genetic screening method using pooled samples analysed by
88 digital PCR (ddPCR) followed by demultiplexed real-time PCR. We amplified one nuclear
89 and two mitochondrial genes using newly designed primers targeting specific variants. We
90 found a low prevalence of MtrBTN2 (23/1516), with only 9 of the 76 sites of the study area
91 affected, including 7 ports (the commercial port, 4 of the 16 marinas and 2 of the 3 landing
92 docks). In contrast to our previous report [14], *M. galloprovincialis* were equally affected,
93 possibly because they coexist closely with *M. edulis* in this region. Interestingly, one site had
94 a high number of MtrBTN2 cases (8/20) and appeared to be a possible hotspot. Given that
95 this site is a landing pontoon for maritime shuttles with frequent shipping traffic to other
96 ports of the area, it could be a source of propagation. A few cancers were found in natural

97 beds near the hotspot, suggesting spillover, although none were found in sites outside other
98 more enclosed ports where MtrBTN2 was detected. In addition, mussel farms and buoy
99 samples were all MtrBTN2-free. These observations suggest that ports could be favourable
100 habitats for MtrBTN2 transmission and/or that maritime transport could play a role as a
101 vector for the spread of the disease, highlighting the role of ports as epidemiological hubs for
102 MtrBTN2 propagation.

103

104 **2. Materials and methods**

105

106 **2.1. Sampling**

107 We collected 1516 *Mytilus* spp. mussels from 76 sites of the French Atlantic coast, from the
108 Bay of Quiberon to Pornic, between January and March 2020. We collected hemolymph from
109 adductor muscle (with a 1 ml syringe, 26G needle) and a piece of mantle; both samples were
110 fixed with 96% ethanol as described in Hammel et al. [14]. DNA extraction was performed
111 with the NucleoMag 96 Tissue kit (Macherey-Nagel) using a Kingfisher Flex extraction robot
112 (serial number 711-920, ThermoFisher Scientific). We followed the kit protocol with a
113 modified lysis time of 1 hour at 56°C and modified the volumes of some reagents: beads
114 were diluted twice in water, 200 µl of MB3 and MB4, 300 µl of MB5 and 100 µl of MB6.
115 DNA concentration (ng/µl) was quantified using the Nanodrop 8000 spectrophotometer
116 (ThermoFisher Scientific). DNA from hemolymph was used for MtrBTN2 detection and
117 DNA from mantle was used for mussel genotyping (Figure S1).

118

119 **2.2. MtrBTN2 detection**

120 As the prevalence of MtrBTN2 is expected to be low [14, 23] and the number of samples is
121 high (n = 1516), we chose to pre-screen by pooling 12 samples (pooled screening step; Figure
122 S1) and then demultiplexing positive pools to specifically target cancerous samples (simplex
123 screening step; Figure S1). Prior to pooling, DNA concentrations were adjusted to 10 ng/µl to
124 obtain an unbiased representation of each sample in the pool. This allowed us to considerably
125 reduce the cost and time of detection, but we acknowledge that this protocol could result in
126 some very early stages of the disease being missed. To partly circumvent this problem,
127 MtrBTN2 detection in pools was performed by digital PCR (ddPCR) targeting one nuclear
128 (Elongation Factor, EF) and one mitochondrial (Control region, described in Yonetmitsu et
129 al. [9]) marker (Table S1). ddPCR is a sensitive method, based on the Taqman method which

130 requires two primers and one probe, and which directly estimates the copy number of the
131 targeted sequence. In addition, the use of a mitochondrial marker should provide a more
132 sensitive detection as the mitochondrial genome has more copies than the nuclear genome.
133 ddPCR primers design and analysis was subcontracted to the company IAGE using the
134 QIAcuity™ system. Positive pools were then demultiplexed (simplex screening step; Figure
135 S1) and MtrBTN2 detection was performed by real-time PCR targeting one nuclear
136 (Elongation Factor, EF1 α i3) and one mitochondrial (cytochrome c oxidase I, mtCOI-sub)
137 marker (Table S1). We used sequences of *M. edulis*, *M. galloprovincialis*, *M. trossulus*, and
138 MtrBTN2 available from the National Center for Biotechnology Information (NCBI) to
139 design the primer pairs (EF1 α i3 and mtCOI-sub). Amplification of both markers was
140 performed using a three-step cycling protocol (95°C for 10 s, 58°C for 20 s, 72°C for 25 s)
141 for 40 cycles. We carried out real-time PCRs using the sensiFAST SyBR No-ROX Kit
142 (Bioline) and the LightCycler 480 Real-Time PCR System. We also confirmed the positive
143 samples diagnosed by real-time PCR using ddPCR (simplex screening step; Figure S1). The
144 specificity of both method and markers was tested using various control samples of *M. edulis*,
145 *M. galloprovincialis*, *M. trossulus*, and MtrBTN2 samples of early, moderate, or late stages
146 (based on cytological observations, following Burioli et al. [23], Tableau S2). To test the
147 sensitivity, we used positive controls with MtrBTN2 DNA diluted 10-fold (x10) and 100-fold
148 (x100) in *M. edulis* DNA.

149

150 Real-time PCR results were analysed using the LightCycler480 software. Two parameters
151 were extracted: threshold cycle (CT), obtained by Absolute Quantification analysis using the
152 Second Derivative Maximum method, and melting temperature (Tm), obtained by Tm calling
153 analysis. Positive samples were defined as those with a CT of less than 35 cycles and a Tm
154 between 77.46 to 78.71 (based on Tm values of the positive control samples; see result,
155 Figure S2). ddPCR results were analysed with the QuantaSoft™ Analysis Pro software,
156 which provides the copy number for each sample. Samples were considered positive when
157 there were more than two positive droplets.

158

159 **2.3. Mussel genotyping**

160 We used 10 biallelic SNPs known to discriminate between *M. edulis* and *M. galloprovincialis*
161 mussel species (Table S3). These markers were identified as being ancestry-informative
162 (fixed for alternative alleles in *M. edulis* and *M. galloprovincialis*) in the Fraïsse et al. dataset
163 [24] and were subsequently confirmed as near diagnostic by analysis of larger datasets [20,

164 25]. Genotyping was performed using the Kompetitive Allele Specific PCR (KASP) method
165 [26, 27]. As we wanted to know the mean ancestry of each sample (G ancestry) and reduce
166 the cost of genotyping, we developed a multiSNPs marker by multiplexing the 10 SNPs.
167 Rapidly, 1 μ L of assay mix (KASP-TF V4.0 2X Master Mix, 1X target concentration;
168 primers, 1 μ M target concentration; HyClone™ HyPure water) and 0.5 μ L of DNA at 10
169 ng/ μ L were mixed in qPCR 384-well plates using Labcyte Echo525. KASP analysis was
170 performed on the LightCycler 480 Instrument (ROCHE) with the following thermal cycling
171 conditions: initialisation 15 min at 95°C, first amplification 20 s at 94°C and 1 min at 61°C to
172 55°C with steps of 0.6°C for 10 cycles, second amplification 20 s 94°C and 1 min at 55°C for
173 29 cycles, read 1 min at 37°C and 1 s at 37°C.

174 KASP results are a combination of two fluorescence values, one for allele X, and another for
175 allele Y. Here, we oriented all SNPs so that allele X corresponds to *M. edulis* and allele Y to
176 *M. galloprovincialis*. Following Cuenca et al. [28], we transformed the data to obtain a single
177 measure of the relative fluorescence of the two alleles, using the following formula:

$$178 \quad y_{i,j} = Y_{i,j} / (X_{i,j} + Y_{i,j}),$$

179 where $X_{i,j}$ and $Y_{i,j}$ are the fluorescence value of allele X and allele Y respectively for i^{th} SNP
180 of the j^{th} sample. To scale $y_{i,j}$ value from 0 when *M. edulis* allele fluorescence dominates at
181 the 10 SNPs to 1 when *M. galloprovincialis* allele fluorescence dominates, we used the
182 following formula:

$$183 \quad y'_{i,j} = (y_{i,j} - \min(y_i)) / (\max(y_i) - \min(y_i)),$$

184 where $y_{i,j}$ is the relative fluorescence value for the i^{th} SNP of the j^{th} sample. In the results
185 section and figures, $y'_{i,j}$ corresponds to “G ancestry”.

186

187 **2.4. Environmental data**

188 The 76 sampling sites were selected to present contrasting environments and genetic
189 backgrounds (*M. edulis*, *M. galloprovincialis* and hybrids). The description of environmental
190 data considered in this study is reported in Table S4 and all site information is available in
191 Table S5. During sampling, we collected several habitat discrete descriptive variables: type of
192 site (port, natural bed, farm, buoy), population density (1: isolated, 2: clustered, 3: beds), and
193 wave action (1: exposed, 2: sheltered). We used the E.U. Copernicus Marine Service
194 Information (doi: [10.48670/moi-00027](https://doi.org/10.48670/moi-00027)) to access temperature (°C), salinity (PSU 1e-3) and
195 current velocity (m.s-1) data for each site (monthly recorded from 2021, resolution of
196 0.028°x0.028°). We also used EMODnet Human Activities (source: EMSA Route Density

197 Map) to access the maritime traffic density of fishing, passenger, and recreational boats in the
198 studied area (seasonal record from 2018, 2019 and 2020, resolution 1x1 km). In the analysis
199 we used the annual average of salinity, current and boat density (maritime traffic). For
200 temperature, we used two measures: the average of winter and autumn temperatures
201 (TempW) and the average of summer and spring temperatures (TempS) as we identified
202 opposite effects between winter and summer temperatures that would be misinterpreted if
203 only the annual average temperature was used (Figure S3).

204

205 ***2.5. Statistical analysis: relationship between MtrBTN2 prevalence, host genotypes and*** 206 ***environmental variables***

207 All statistical analyses were performed in R v4.1.3 [29]. Fisher's exact tests were used for the
208 analysis of contingency tables of cancer prevalence (*fisher.test()* function of the *stats* package
209 v4.1.3 [29]). A principal component analysis (PCA) was used to explore the relationship
210 between the quantitative environmental variables (*PCA()* function of the *FactoMineR*
211 package v2.6 [30]; missing values were estimated using *imputePCA()* of the *missMDA*
212 package v1.18 [31]). The association of MtrBTN2 prevalence with each environmental
213 variable (Table S4) was tested using a univariate Poisson regression (*glm()* function of *stats*
214 package v4.1.3 [29]). Significant variables (p-value < 0.05) were selected and included in a
215 multivariate Poisson regression. We also used redundancy analysis (RDA) to assess the
216 overall relationship between cancer diagnosis and environmental variables. Real-time PCR
217 and ddPCR results for both markers were synthesised to define positive samples: negative
218 samples had 0 and cancerous samples had CT or copy number values (1516 x 4 matrix). All
219 environmental data described above, including site types, were used as explanatory variables
220 (*rda()* function of the *vegan* package v2.6-4 [32]). Forward selection using the *ordiR2step()*
221 *vegan* function was then used to determine the significance of the variables.

222

223 **3. Results**

224

225 ***3.1. Validation of the screening method***

226 In order to detect MtrBTN2 in thousands of individuals, we developed dedicated ddPCR and
227 qPCR screening tools.

228 The design of real-time PCR and ddPCR primers and probes allowed us to amplify nuclear
229 and mitochondrial loci specific to *M. trossulus* mussels and MtrBTN2 (Figure S4, TableS6).

230 However, for the mtCOI-sub locus, the melting temperature (T_m) range of MtrBTN2 was

231 different from *M. trossulus* controls and allowed us to specifically detect MtrBTN2 based on
232 Tm values (Figure S2).

233 With ddPCR, all MtrBTN2 control samples were detected, regardless of marker or dilution,
234 except for one MtrBTN2 sample diluted at x100 which was negative for the EF locus (Figure
235 S4). Copy numbers appeared higher for the mtCR marker, showing a higher sensitivity for
236 this mitochondrial marker. The same pattern was observed for EF1 α -i3 and mtCOI-sub in
237 real-time PCR, with a higher sensitivity of the mitochondrial marker based on the lower CT
238 values observed (Figure S4). However, two MtrBTN2 samples diluted at x100 were negative
239 for mtCOI-sub and positive for EF1 α -i3. Even though the mitochondrial marker appears to be
240 more sensitive in both methods, we chose to screen for MtrBTN2 using both markers in order
241 to have comparisons and additional validation.

242

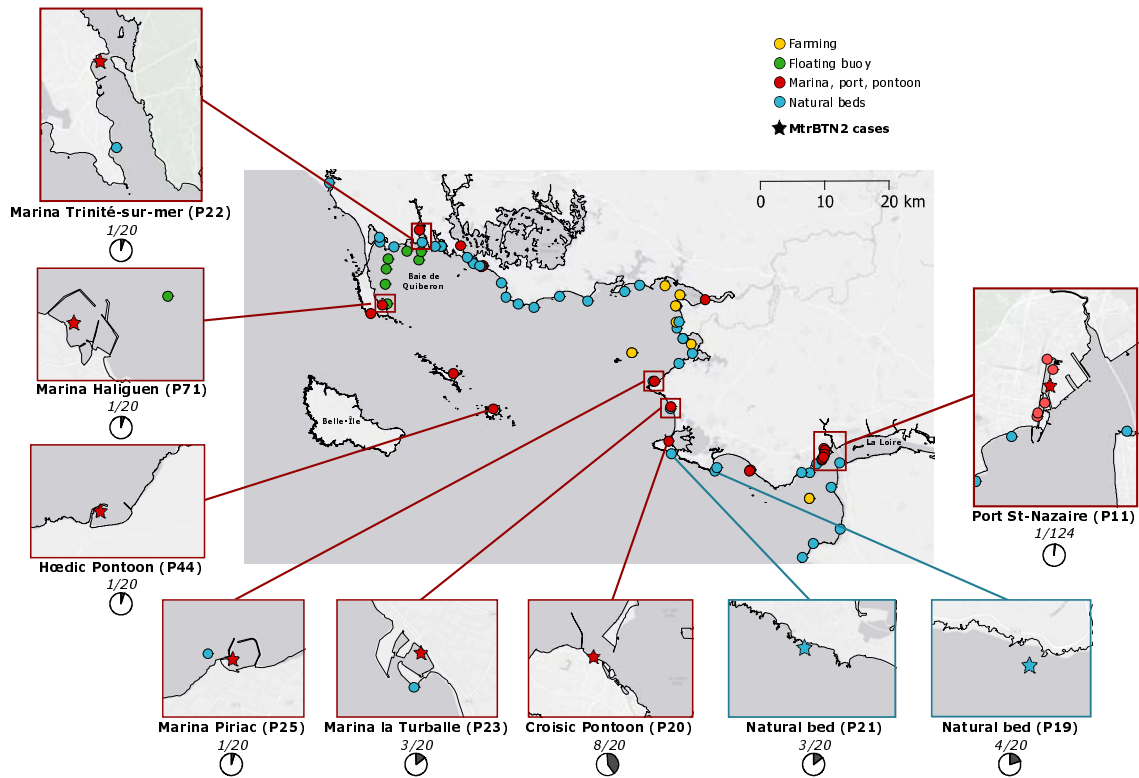
243 **3.2. MtrBTN2 screening reveals a low prevalence**

244 We amplified nuclear and mitochondrial markers using ddPCR (EF, mtCR) and real-time
245 PCR (EF1 α -i3, mtCOI-sub) to detect the presence of MtrBTN2 in the 1516 mussels sampled
246 from the 76 sites of the study area (Figure S1). All results of the pooled and simplex
247 screening are presented in TableS7.

248 The pooled screening step using ddPCR revealed 25/127 positive pools: 9/127 with EF,
249 14/127 with mtCR, and 2/127 were positive for both markers (Figure S4). As the 14 mtCR-
250 positive pools showed low copy numbers (median = 7.59), we assume that EF did not
251 amplify because of a detection threshold. For pools positive for both markers, the EF copy
252 numbers appear relatively low compared to mtCR copy numbers. This is due to the high
253 mitochondrial fluorescence masking the nuclear fluorescence and underestimating EF copy
254 numbers. To eliminate this bias in the simplex ddPCR step (Figure S1), we amplified the two
255 markers separately. In order to maximise cancer detection in the simplex screening step, we
256 chose to keep the pools positive for at least one gene, corresponding to 25 positive pools of
257 12 samples (n = 300).

258 We performed the simplex screening step using real-time PCR and then confirmed positive
259 samples with ddPCR (Figure S1). In total, 44/300 samples were positive for at least one real-
260 time PCR marker: 16 with EF1 α -i3 only, 23 with mtCOI-sub only, and 5 with both markers
261 (Figure S4, Table S8). Pooled and simplex screening results are mostly consistent, as 20 of
262 the 25 positive pools had at least one individual detected by real-time PCR amplification
263 (Table S9). The 5 pools without positive samples had low ddPCR copy numbers (less than
264 3.37 for EF and less than 6.65 for mtCR). These false positives were to be expected as we

265 intentionally applied low threshold values in the pooled screening step. Two samples from
266 mtCR-positive pools were found to be positive for both markers at the simplex level, which
267 was expected as the mitochondrial marker is likely to be more sensitive than the nuclear
268 marker. On the contrary, we did not expect to observe samples positive for EF1 α -i3/EF and
269 negative for mtCOI-sub/mtCR. However, all but one of the individuals from the EF-positive
270 pools were positive only for the EF1 α -i3 marker (Table S9). The absence of mitochondrial
271 genes in these samples does not seem to be related to a detection threshold issue, especially
272 as ddPCR and real-time PCR target two different genes (mtCR and mtCOI-sub). We suspect
273 that some host alleles that were present at low frequencies in the ‘dock mussels’ populations
274 (the hybrid lineage found in the port of Saint-Nazaire [25]) may sometimes be amplified with
275 EF1 α -i3. This could be explained by shared polymorphism between host species rather than
276 the presence of MtrBTN2. Therefore, the 16 samples positive only for EF1 α -i3 were
277 conservatively considered as false positives. For the remaining 28 samples, we performed
278 ddPCR with both markers to confirm the real-time mitochondrial PCR diagnosis (Figure S1,
279 TableS8). For 23/28 samples, mtCR ddPCR confirmed the mtCOI-sub real-time PCR
280 diagnosis. For 10/23 samples positive only for mtCOI-sub, ddPCR revealed few EF copies.
281 Finally, we considered all samples positive for mitochondrial markers with both methods to
282 be cancerous samples. We found 23/1516 positive samples from 9/76 sites in which the
283 number of cases varied from 1 to 8 (Figure 1). The highest proportion of cancerous mussels
284 was found at site P20 (Croisic Pontoon), and the next highest in the vicinity of this site (P19,
285 P21, and P23; Figure 1).
286



287

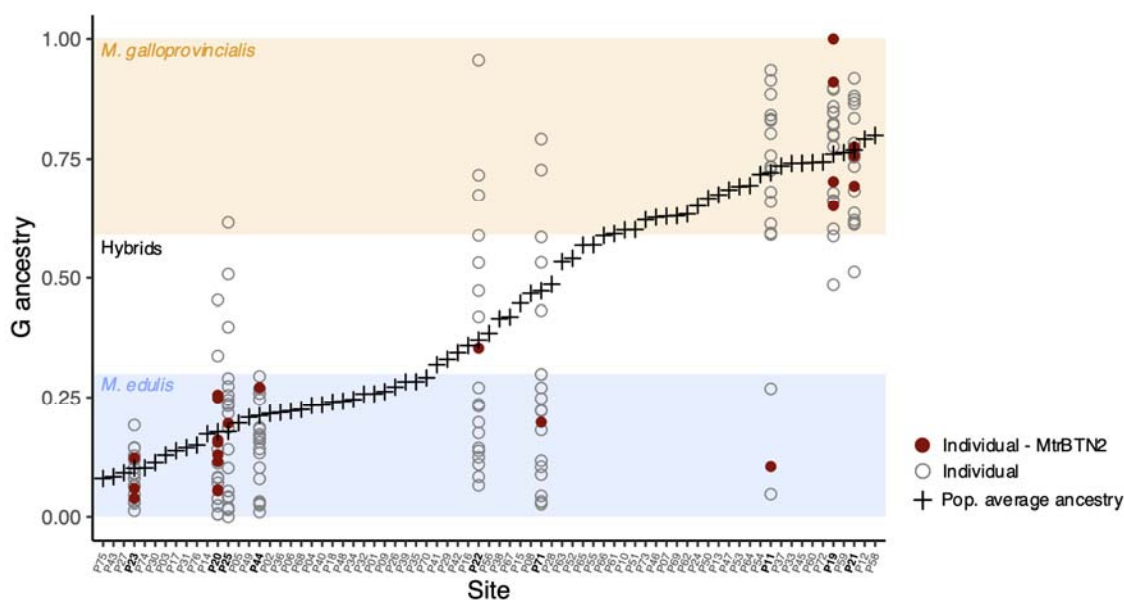
288 **Figure 1: Location of sampling sites.** Zoomed-in sites correspond to sites where mussels are
289 affected by MtrBTN2. Pie graphs and numbers in italics correspond to the proportion of
290 cancerous samples in each site. Coloured points represent the type of site, as indicated in the
291 figure's legend. Map origin: Ersi gray (light).
292

293 3.3. *M. edulis* and *M. galloprovincialis* are equally affected by MtrBTN2

294 To determine whether host genetic background or population composition influences cancer
295 prevalence and distribution, mussels were genotyped using 10 SNPs known to be diagnostic
296 between *M. edulis* and *M. galloprovincialis* (the two species coexisting in the studied area).

297 To reduce the cost of genotyping, we developed a multiplex assay, mixing 10 SNPs that
298 directly give the average ancestry of the sample (G ancestry, Figure S1). This approach was
299 validated by comparing the multiplex fluorescence and average simplex fluorescence of
300 SNPs from a set of samples (Figure S5a). We genotyped each individual from the 9 sites
301 affected by MtrBTN2 ($9 \times 20 = 180$ mussels) and estimated the average ancestry of each site
302 by genotyping pools of individuals (76 pools of 20 mussels). The good positive correlation
303 between the average individual fluorescence of a site and the fluorescence of the
304 corresponding pool validated our approach (Figure S5b). Mussels with G ancestry values
305 below 0.3 were assigned to *M. edulis*, those with values above 0.59 to *M. galloprovincialis*,
306 and those in between were assigned as hybrids (Figure 2, FigureS6). Of the mussels affected

307 by MtrBTN2, 15/23 were assigned to *M. edulis*, 7/23 to *M. galloprovincialis* and 1/23 was a
308 hybrid (Table S10). Considering the sites affected by MtrBTN2, MtrBTN2 prevalence did not
309 appear to be significantly different between species with 15/105 (14%) in *M. edulis*, 7/60
310 (11%) in *M. galloprovincialis* and 1/15 (6%) in hybrids (Fisher exact test, p-value = 0.83).
311 Most *M. edulis* mussels affected by MtrBTN2 were found in populations with a majority of
312 *M. edulis* (P44, P23, P20), only two were found in populations with some hybrids and *M.*
313 *galloprovincialis* mussels (P25, P71) and only one in a population with a majority of *M.*
314 *galloprovincialis* (P11, Figure 2, Figure S6). Interestingly, all *M. galloprovincialis* mussels
315 affected by MtrBTN2 were found in *M. galloprovincialis* populations (P19, P21, Figure 2,
316 FigureS6), which are close to the site with the highest prevalence (Croisic pontoon, Figure 1).
317 Finally, the hybrid individual affected by MtrBTN2 was found in a mixed population (P22,
318 Figure 2, FigureS6).
319



320

321 **Figure 2: Individual and population ancestry.** G ancestry of healthy (empty grey circle)
322 and MtrBTN2 affected (dark red) hosts is shown for the 9 sites with cancer (bold labels).
323 Population average ancestry for all sampled sites is represented by black crosses. Blue, white
324 and orange rectangles correspond to G ancestry range of *M. edulis*, hybrids and *M.*
325 *galloprovincialis* respectively.
326

327

328 **3.4. MtrBTN2 infected mussels are mostly found in ports with high density of maritime traffic**

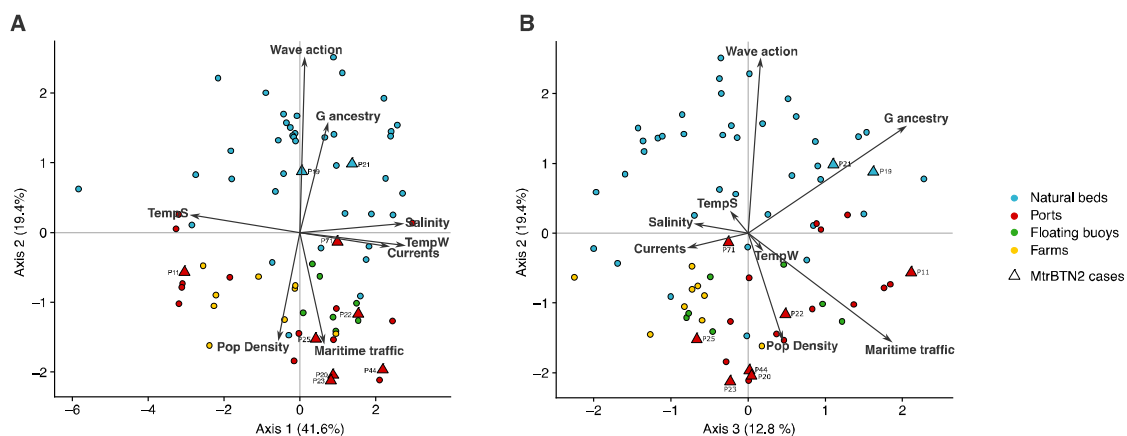
329

329 After the MtrBTN2 detection step, which revealed 23/1516 individuals infected with this
330 transmissible cancer, we investigated associations with environmental factors. Cancerous

331 samples were found in 7 ports (16/23 infected mussels) and 2 natural beds (7/23) near the
332 Croisic pontoon (P20, Figure 1). MtrBTN2 was not detected in the 180 individuals from the 9
333 mussel farms or in the 140 individuals from the 7 floating buoys. Cancerous samples were
334 significantly more frequent in ports than in other types of sites (Fisher exact test, p -value =
335 0.0001, Table S11).

336 The PCA analysis reveals an association between environmental variables and the types of
337 sites rather than the presence of cancer itself (Figure 3). Indeed, ports, floating buoys and
338 farms are well separated from natural beds on the first factorial plan while similarly found in
339 sheltered habitats with high population density. Interestingly, maritime traffic (i.e., annual
340 mean of passenger, fishing, and recreational boat density) tends to explain a direction of
341 variation towards human-altered habitats, but also towards sites affected by MtrBTN2.
342 MtrBTN2 seems to be found in sites with cooler mean temperatures in summer and warmer
343 mean temperatures in winter and high mean annual salinity and current velocity,
344 environmental variables that characterise ports. Water temperature tends to be buffered in
345 ports, being warmer in winter and cooler in summer (Figure S3). However, these variables
346 are also difficult to interpret as they also explain well the inertia of axis 1 of the PCA which
347 differentiates the sites located near estuaries (La Loire or La Vilaine) from the others. To
348 better identify the environmental variables that best explain MtrBTN2 prevalence despite
349 being correlated, we used an ordination method (RDA) and a generalized linear model
350 (GLM).

351



352

353 **Figure 3: PCA on environmental variables.** (A) axis 1 versus axis 2, (B) axis 2 versus axis
354 3 of the PCA analysis.

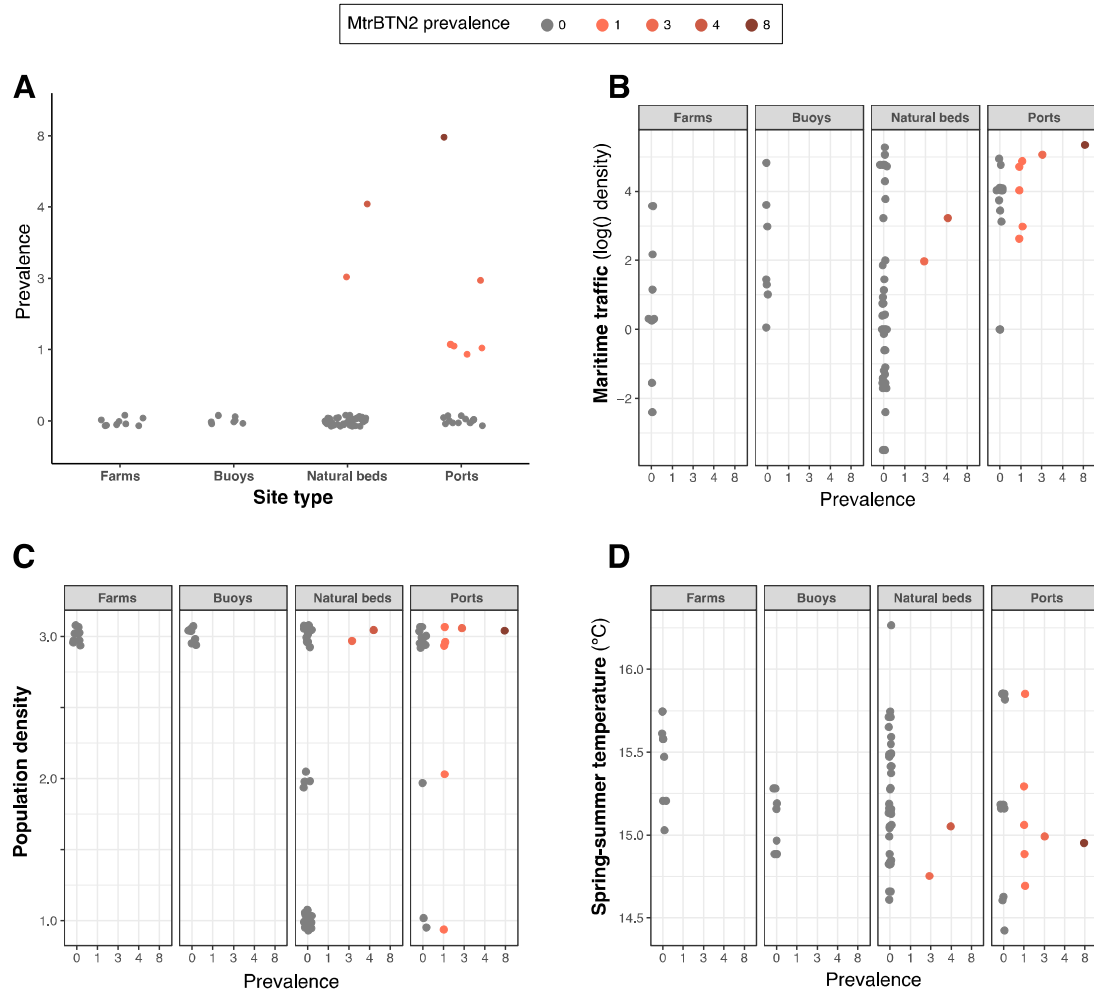
355

356 The RDA analysis explores the statistical association between the presence of cancer and
357 environmental variables, including types of sites. Of the 9 environmental variables included
358 in our study (Figure S7), 4 were found to be significant in explaining the presence of cancer
359 after bootstrapping: maritime traffic (p-value = 0.002, R^2_{adj} = 0.013), population density (p-
360 value = 0.012, R^2_{adj} = 0.015), site type (p-value = 0.02, R^2_{adj} = 0.020) and tempS (spring-
361 summer temperature; p-value = 0.02, R^2_{adj} = 0.022). It is worth noting that, according to the
362 PCA observation, tempS may explain the influence of estuary water and buffered temperature
363 in ports rather than the presence of cancer itself.

364 The multivariate Poisson GLM on cancer prevalence was conducted on the four numerical
365 variables that significantly explained the prevalence of cancer in the univariate models
366 (maritime traffic, population density, spring-summer temperature (tempS) and currents, Table
367 S12). Categorical data on site type were not included in this analysis. At the multivariate
368 level, the prevalence of cancer was significantly explained by maritime traffic (p-value =
369 0.002) and population density (p-value = 0.026, Table S12), in agreement with the RDA.

370 The univariate correlations of the four variables that best explain MtrBTN2 prevalence in the
371 ordination and GLM analyses, namely maritime traffic and population density, as well as site
372 type and tempS, are presented in Figure 4.

373



374

375 **Figure 4: Univariate correlations of environmental variables that best explain the**
376 **prevalence of MtrBTB2: site type (A), maritime traffic (B), population density (C) and**
377 **spring-summer temperature (D). Coloured points correspond to the prevalence of MtrBTN2**
378 **as indicated in the legend.**

379

380 4. Discussion

381

382 Our results show that MtrBTN2 is present at low prevalence along the French Atlantic coast
383 ($23/1516 = 1.5\%$). Strikingly, it is mostly found in ports and is equally distributed in *M.*
384 *edulis* and *M. galloprovincialis*. Our analysis of environmental factors influencing the
385 epidemiology of MtrBTN2 shows that ports are likely epidemiological hubs for MtrBTN2
386 propagation.

387

388 Most MtrBTN2-infected mussels were found in ports and a hotspot was found around the
389 Croisic pontoon. Given the low prevalence of MtrBTN2 previously described [14, 23] and

390 confirmed in this study, we acknowledge that our sample sizes per site may be too small (n =
391 20) to assess MtrBTN2 prevalence with confidence at a site level. However, our careful
392 overall sampling design, capturing replications of habitat features, has allowed us to test for
393 correlations between environmental factors and uncover higher prevalence of MtrBTN2 in
394 ports.

395

396 The highest prevalence of MtrBTN2 in ports could be explained by two non-exclusive
397 hypotheses. Firstly, ports could offer a favourable environment as it is conducive to disease
398 development and propagation. Indeed, ports are often polluted, confined and permanently
399 immersed habitats (without tidal constraints) with high density of mussels. Please consider
400 that our objective here is not to explain the emergence of a transmissible cancer, but its
401 maintenance and spread. The emergence probably took place long before the industrial period
402 and the mutagenic effect of pollutants does not have to be put forward to explain our results,
403 but rather their effect on host immunity. Secondly, there is increased connectivity between
404 ports due to maritime traffic and ports are connected to other sites (other ports or wild sites)
405 through biofouling from vessels.

406

407 Pollution is a major environmental stressor that could affect host physiology and increase
408 susceptibility to parasites, particularly by altering the immune response (reviewed in [33]).
409 As filter-feeders, species of bivalve molluscs are particularly concerned [34–37]. However, in
410 our study, most of the affected individuals (15/23) were found in three open sea sites known
411 to be healthy and protected ecosystems with abundant resources (Croisic Pontoon, Natural
412 beds 19 and 21 part of Natura 2000 area; Figure 1), suggesting that pollution alone cannot
413 explain the higher prevalence of MtrBTN2 cancers in ports. Conversely, it can be
414 hypothesised that a healthy, less stressful environment may increase the host permissiveness
415 and allow MtrBTN2 (and possibly other BTNs) to persist at a higher detectable prevalence.
416 Host nutrition can affect disease outcome by driving host immunity and parasite resource
417 availability [38–40]. In invertebrates, increased host nutrition can lead to increased parasite
418 load [41] and improved immunity against parasites [39, 40]. This balance could apply to
419 mussel-MtrBTN2 interaction, and a healthy environment with abundant resources could
420 increase host carrying capacity and/or slow down disease progression. A polluted
421 environment could also affect MtrBTN2 cells survival in seawater and reduce their chances
422 of infecting a new host. However, the impact of pollution on host and cancer cells needs to be

423 further investigated to clearly establish its influence on the transmission and persistence of
424 such transmissible cancers.

425

426 Ports are often confined areas with long particle residence times which could increase the
427 contact time between parasites and hosts, thereby increasing the probability of transmission
428 and favouring the persistence of a passively dispersing parasite. However, the major hotspot
429 on Croisic Pontoon, which is affected by strong tidal currents, again provides evidence
430 against this hypothesis given that it is not a confined area.

431

432 Host population density is an important epidemiological parameter, as high density is known
433 to favour parasite persistence in host populations [42–44]. Our results suggest that highest
434 MtrBTN2 prevalence rates are found in sites with high population density. However, we
435 found no evidence of MtrBTN2 cases in mussel farms despite high mussel population density
436 in these sites. Although population density could favour MtrBTN2 persistence in an affected
437 population, this parameter alone cannot explain the higher prevalence of MtrBTN2 in ports.

438

439 Connectivity through maritime traffic appears to be the main common feature between
440 affected ports. It is conceivable that certain hotspots exist at certain locations along the coast,
441 either due to environmental factors that have yet to be characterised, or simply due to
442 stochastic local outbreaks. If a port is affected by such a local outbreak, it will become a
443 source of contamination to other ports through biofouling of vessels, while natural beds will
444 probably remain isolated by density troughs that could halt the propagation (Figure 5). While
445 this does not disqualify the factors discussed above – e.g., pollution, population density and
446 confinement- as drivers of contamination within ports, our study suggests that maritime
447 routes between ports could be anthropogenic gateways to MtrBTN2 propagation.
448 Furthermore, despite a high density of maritime traffic around the floating buoys we sampled,
449 these sites remained free of MtrBTN2. This could indicate that in addition to cruising, ships
450 must dock for the spread of MtrBTN2 to be effective, as is the case in ports (Figure 5). This
451 result represents a significant advance in our understanding of MtrBTN2 epidemiology, both
452 by proposing ports as epidemiological hubs and by suggesting that the natural propagation of
453 this transmissible cancer could be naturally halted by density troughs in the distribution of
454 host mussel populations. *M. galloprovincialis* was thought to be more resistant to MtrBTN2
455 because of lower prevalence in this species [14], and patches of *M. galloprovincialis* could
456 also have acted as barriers to disease propagation between patches of *M. edulis*. However, our

457 results suggest that *M. galloprovincialis* population patches close to *M. edulis* populations
458 may also show enhanced prevalence. This does not contradict the idea that *M.*
459 *galloprovincialis* population patches could impose a slightly higher resistance to MtrBTN2
460 propagation. Under the hypothesis that natural propagation is hindered by density troughs
461 between population patches, or by population patches of a more resistant species, ports and
462 ship traffic would create anthropogenic gateways that favour connections between patches of
463 host populations (Figure 5). This hypothesis would also suggest that BTNs spread naturally
464 and progressively from one host to another by close contact rather than drifting with the
465 currents, making enhanced R0 by anthropogenic dispersal a threat to host populations.
466



468 **Figure 5: Diagram illustrating the discussion on the propagation of MtrBTN2 across**
469 ***Mytilus* sp. populations.** Arrows widths correspond to the probability of MtrBTN2
470 propagation. Blue and orange mussels correspond to *M. edulis* and *M. galloprovincialis*,
471 respectively. Diagram created with BioRender.
472

473 A hotspot of MtrBTN2 prevalence was found in the area around the Croisic pontoon. This
474 specific site could represent a source for other ports and surrounding natural beds. Indeed,
475 this pontoon is frequently used by maritime shuttles, as well as by recreational and fishing
476 boats, and is connected by maritime traffic to many ports in the studied area. In particular,
477 this pontoon is the departure site of boats travelling to Hoedic (site P44 in Figure 1), an
478 isolated island for which the presence of cancer is most likely explained by anthropogenic
479 transport given the distance from the mainland. MtrBTN2 is also found in foreshores near
480 Croisic. This reveals a possible spillover from ports to adjacent wild populations (Figure 5).
481 Maritime traffic has been identified as a vector for non-indigenous species, including their
482 expansion from commercial ports to natural populations via marinas [45–48]. It has also been
483 documented that biofouling from ships can introduce parasites into unaffected areas and have
484 secondary impacts on local populations, including causing significant mortality (see [49]). In
485 our study, when sites outside standard enclosed ports (e.g., enclosed marinas) were sampled,

486 MtrBTN2 was never detected, suggesting that contagion remains confined to the port area.

487 This suggests that spillover may depend on the degree of confinement of a port.

488

489 **5. Conclusion**

490

491 Our study reveals that the prevalence of MtrBTN2 along the South Brittany French coast is
492 low, but that the most affected sites are ports (marinas, commercial ports, and docks). This
493 result suggests that ports are epidemiological hubs and maritime routes anthropogenic
494 gateways for the propagation of MtrBTN2. Detection of MtrBTN2 presence on boat
495 biofouling would provide more direct evidence of its role as vector. It would also be
496 interesting to investigate whether prevalence hotspots are more commonly located in areas
497 with particular environmental conditions, or whether they are stochastic local outbreaks. This
498 could be done by detecting new hotspots or by sampling Croisic pontoon again in the
499 future. The spreading of (micro)parasites and pathogens through vessel biofouling is a
500 legitimate concern, and results such as ours -i.e., on a smaller scale than translocations
501 identified in the marine bioinvasion literature or by Yonemitsu et al. [9]- highlight the critical
502 need for policy regulation to limit the effects of biofouling, both on ship hulls and port
503 docks, in addition to ballast water control.

504

505 **Data accessibility**

506 All data used in this study are available in the supplementary material.

507 **Author's contribution**

508 M.H., Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Super-
509 vision, Visualization, Writing – original draft, Writing – review and editing. F.To., Investiga-
510 tion, Data curation, Writing – review and editing. E.A.V.B., Formal analysis, Investigation,
511 Writing – review and editing. L.P., Data curation, Investigation, Writing – review and edit-
512 ing. F.C., Investigation, Writing – review and editing. E.G., Investigation, Writing – review
513 and editing. I.B., Resources, Writing – review and editing. H.C., Resources, Writing – review
514 and editing. A.S., Formal analysis, Writing – review and editing. F.Th., Conceptualization,
515 Funding acquisition, Writing – review and editing. D.D-G., Conceptualization, Funding ac-
516 quisition, Writing – review and editing. G.M.C., Conceptualization, Project administration,
517 Funding acquisition, Writing – review and editing. N.B., Conceptualization, Funding acquisi-

518 tion, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writ-
519 ing – original draft, Writing – review and editing.

520

521 **Conflict of interest declaration**

522 We declare we have no competing interests.

523

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528 LABX-0004) and Tulip (ANR-10-LABX-41). FT is supported by the MAVA Foundation.

529

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534

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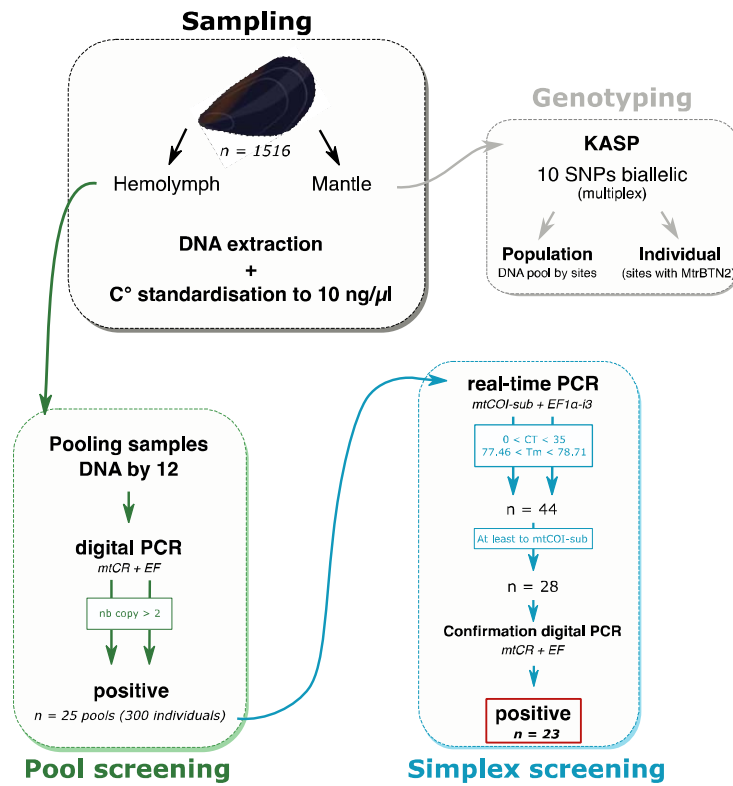
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 659

660

661 **Supplementary figures**

662

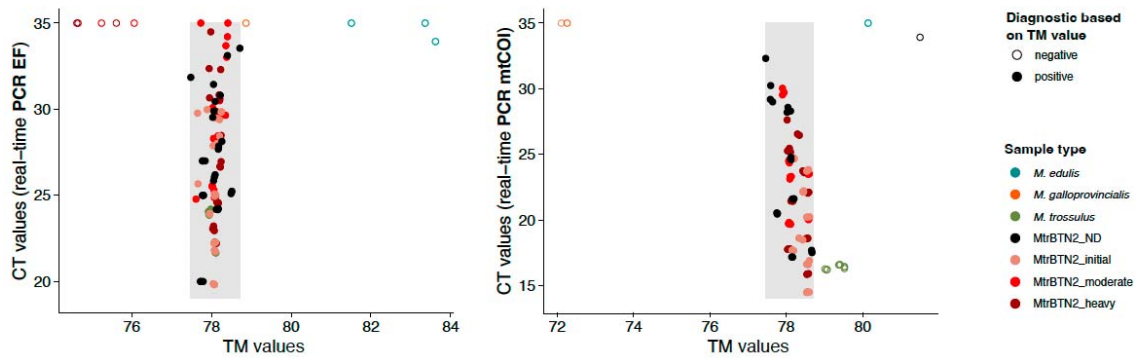


663

664 **Figure S1: Diagram of screening and genotyping experimental steps.**

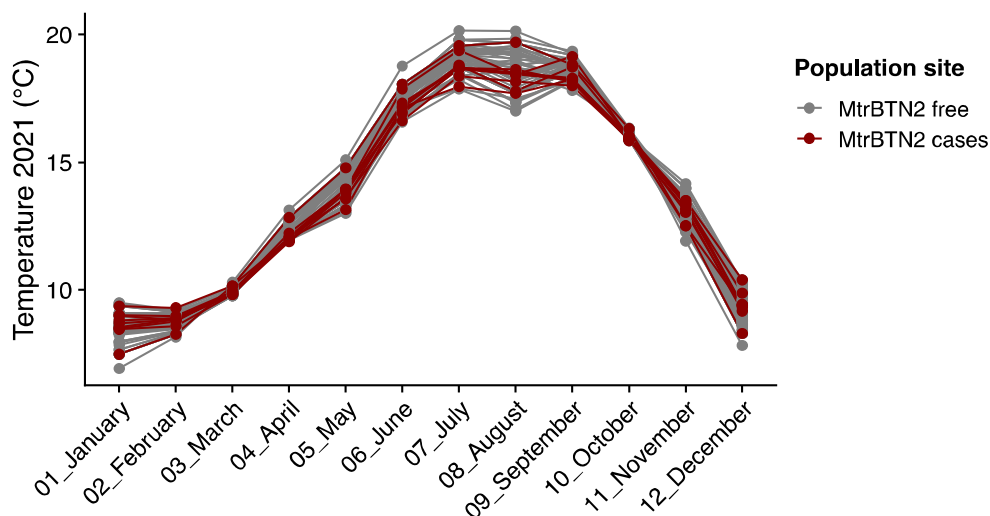
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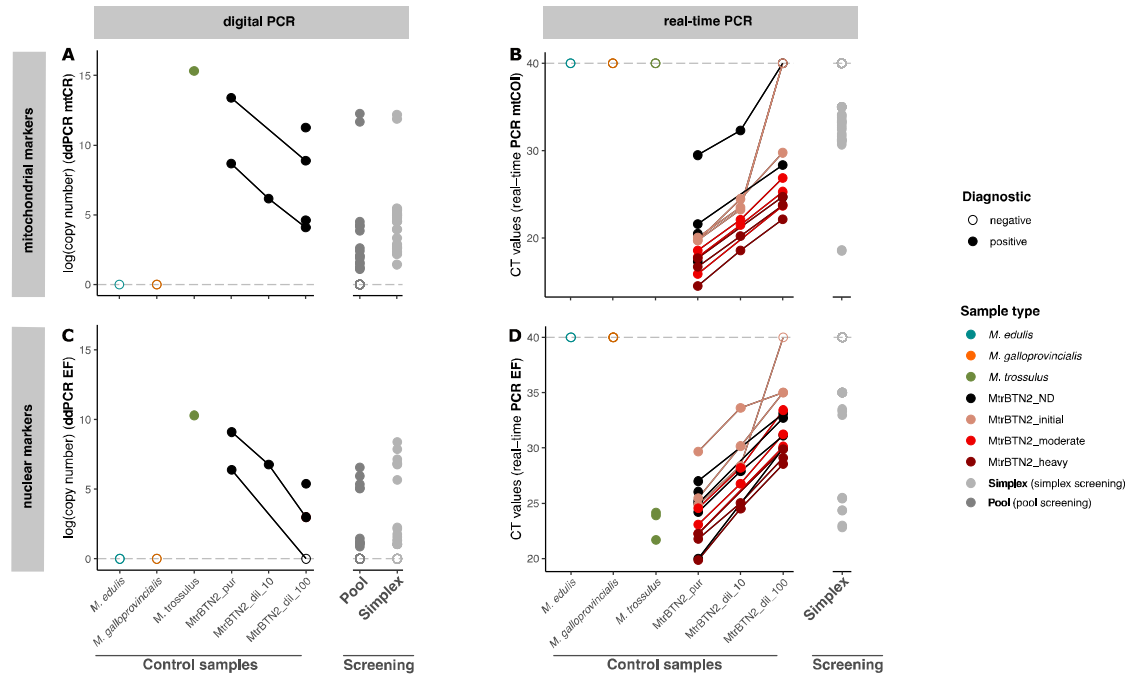


667

668 **Figure S2: TM threshold determination for EF1 α -i3 (left) and mtCOI-sub (right) real-**
669 **time PCR markers.** Grey rectangles correspond to TM threshold based on MtrBTN2
670 positive control samples ($77.46 < TM < 78.71$). Sample type colour and diagnostic based on
671 TM values are indicated in the legend. *M. edulis* and *M. galloprovincialis* correspond to
672 negative controls, *M. trossulus* are *M. trossulus* controls, and MtrBTN2 samples are positive
673 controls (ND: cancer stage not defined, initial: early stage, moderate: moderate stage, heavy:
674 late stage, see TableS2)
675
676
677



678
679 **Figure S3: Sampled sites temperature, recorded monthly in 2021.** Points correspond to a
680 temperature measurement, solid lines refer to MtrBTN2-free sites (grey) and MtrBTN2
681 affected sites (darkred).
682



683

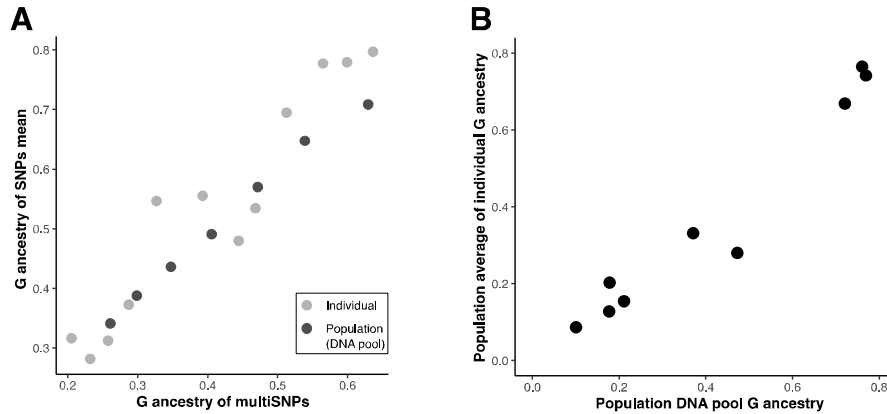
684 **Figure S4: Real-time PCR and digital PCR results.** (A) and (C) represent ddPCR results of EF
 685 and mtCR, and (B) and (D) represent real-time PCR results of EF1 α -i3 and mtCOI-sub,
 686 respectively. Dotted lines correspond to the negative thresholds, 0 for no DNA copy detected by
 687 ddPCR and 40 for no amplification in real-time PCR. Solid lines in each graph refer to the same
 688 sample diluted to different concentrations (pure, x10, x100). *M. edulis* and *M. galloprovincialis*
 689 correspond to negative controls, *M. trossulus* are *M. trossulus* controls, and MtrBTN2
 690 samples are positive controls (ND: cancer stage not defined, Initial: early stage, moderate:
 691 moderate stage, heavy: late stage, see TableS2). Screening results using both method and
 692 markers are shown for Pool and Simplex. Sample type colours and diagnostic status are
 693 indicated in the figure legend.

694

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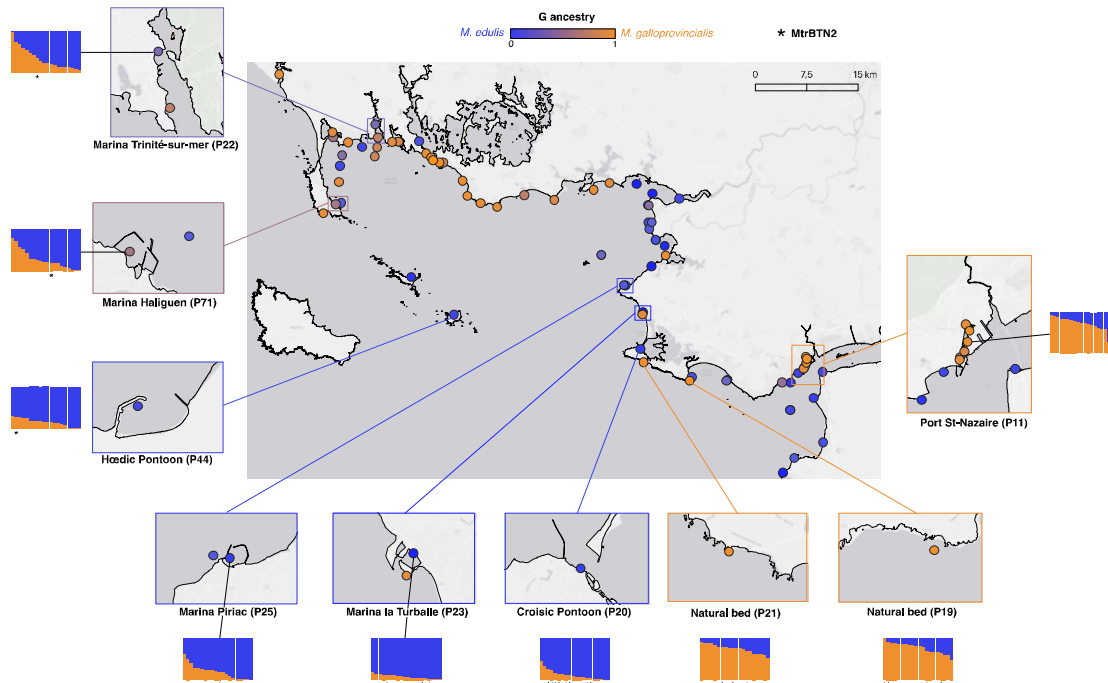
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698

699 **Figure S5: Genotyping method validation.** (A) Positive correlation between multiSNPs G
700 ancestry and the mean of the 10 single SNPs from a subset of Individual and Pool samples.
701 (B) Positive correlation between population DNA pool G ancestry and the mean individual G
702 ancestry of individuals from the same population.

703



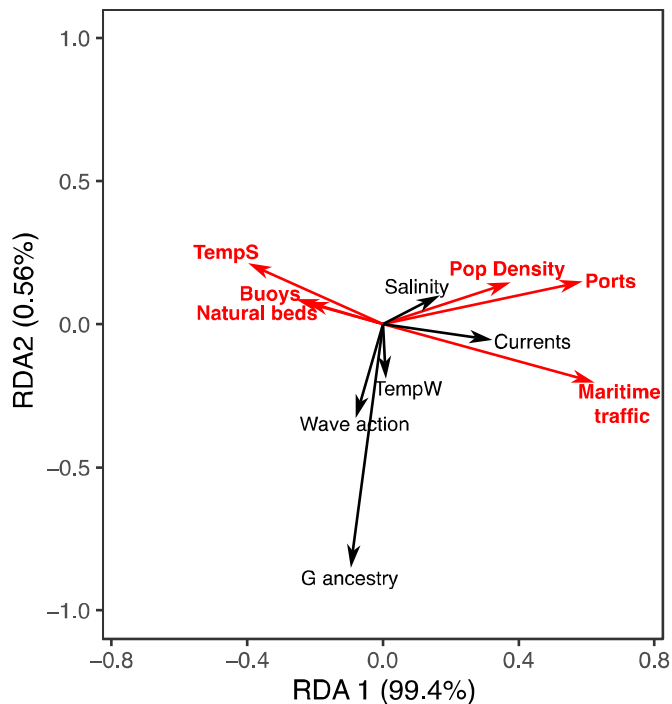
704

705 **Figure S6: Population and individual ancestry composition across the sampled area.**
706 Zoomed-in sites correspond to sites with mussels affected by MtrBTN2. Barplots represent
707 the estimated ancestry of individuals based on the 10SNPs multiplex tool. MtrBTN2
708 individuals are indicated by stars under each barplot. Coloured points represent the
709 population average ancestry, as indicated in the figure's legend. Map origin: Ersa gray (light).

710

711

712



713

714 **Figure S7: RDA analysis to assess overall relationship between presence of MtrBTN2**
715 **affected mussels and environmental factors.** Arrows represent environmental variables.
716 Red arrows correspond to significant variables obtained by forward selection with the
717 `ordiR2step()` function (R software). Axis 1 correlates positively with the presence of
718 MtrBTN2.

719

720

721 **Supplementary Tables (→ [See excel TableS1-12](#))**

722

723 **Table S1: Primer and probe sequences used in real-time PCR and digital PCR.**

724

725 **Table S2: Control samples used to validate real-time PCR and ddPCR screening tools.**

726 All samples are from our laboratory sample collection. *M. edulis* and *M. galloprovincialis*
727 correspond to negative controls, *M. trossulus* are *M. trossulus* controls, and MtrBTN2
728 samples are positive controls. MtrBTN2 stages were defined by cytology of the hemolymph,
729 according to the following classification: early (<15% cancer cells), moderate (15-75%
730 cancer cells), late (>75% cancer cells), ND when cancer stage was not defined.

731

732 **Table S3: Bi-allelic nuclear SNPs used for the multiSNPs genotyping.**

733

734 **TableS4: Source and description of environmental variables.**

735

736 **TableS5: Description of sampled sites. Sources of environmental variables** are provided in
737 TableS4. Site: site name; MtrBTN2 prevalence: number of MtrBTN2 affected mussels;
738 TempS: mean spring-summer temperature; TempW: mean autumn-winter temperature;
739 temp: temperature; sal: salinity; vel: current; [fish-pass-other][19-21]_[1-4], fish: fishing boat
740 density, pass: passenger boat density, other: recreational boat density, 19 to 21 correspond to
741 the year of record, and 1 to 4 correspond to spring, summer, autumn, winter, respectively.

742

743 **Table S6: Control sample results validate real-time PCR and ddPCR screening tools.**
744 EF_CopyNb: copy number for EF ddPCR marker; mtCR_CopyNb: copy number for mtCR
745 ddPCR marker; CT: threshold cycle, TM: melting temperature for EF1 α -i3 and mtCOI-sub
746 real-time PCR markers. NP: not performed.

747

748 **TableS7: Pooled and simplex screening results.** Pool_CopyNb_EF: copy number for EF
749 ddPCR marker (pooled screening); Pool_CopyNb_mtCR: copy number for mtCR ddPCR
750 marker (pooled screening); CT: threshold cycle, TM: melting temperature for EF1 α -i3 and
751 mtCOI-sub real-time PCR markers; Simplex_CopyNb_EF: copy number for EF ddPCR
752 marker (simplex screening); Simplex_CopyNb_mtCR: copy number for mtCR ddPCR
753 marker (simplex screening). NP: not performed.

754

755 **TableS8: Simplex screening results for positive samples.** CT: threshold cycle, TM: melting
756 temperature for EF1 α -i3 and mtCOI-sub real-time PCR markers; PCR_result: marker name
757 positive in real-time PCR; EF_CopyNb: copy number for EF ddPCR marker;
758 mtCR_CopyNb: copy number for mtCR ddPCR marker; ddPCR_result: marker name
759 positive in ddPCR; MtrBTN2: final diagnostic status. NP: not performed.

760

761 **Table S9: Correspondence between pool and simplex screening.** Pool_CopyNb_EF : copy
762 number for EF ddPCR marker (pooled screening); Pool_CopyNb_mtCR : copy number for
763 mtCR ddPCR marker (pooled screening); Pool_ddPCR_result: marker name positive in
764 ddPCR (pool); Nb_ind_positive: number of individuals in the pool found positive for one or
765 two marker in simplex screening; Simplex_PCR_result: marker name of the positive
766 individual in real-time PCR; Simplex_ddPCR_result: marker name of the positive individual
767 in ddPCR, negative if the sample did not amplify. NP: not performed.

768

769 **Table S10: MultiSNPs genotyping results.** G ancestry: fluorescence value; Ancestry:
770 ancestry defined based on G ancestry values.

771

772 **Table S11: Contingency table used for the Fisher exact test.**

773

774 **Table S12: Poisson regression models testing the effect of environmental variables on**
775 **MtrBTN2 prevalence.** Model results were obtained using glm() function in R. Variables
776 explaining MtrBTN2 prevalence at the univariate level were selected for the multivariate
777 model. TempS: spring-summer temperature.

778